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Metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the patas monkey: pharmacokinetics and characterization of glucuronide metabolites

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The metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was examined in the patas monkey, in order to provide further information about NNK metabolic pathways in primates. Female patas monkeys were given i.v. injections of [5-^{3}H]NNK, and metabolites in serum and urine were analyzed by HPLC. Metabolism by α-hydroxylation of NNK was rapid and extensive, and the products of this pathway, 4-hydroxy-4-(3-pyridyl)butyric acid and 4-oxo-4-(3-pyridyl) butyric acid, accounted for a relatively large proportion of serum and urinary metabolites at all time points. This is significant because the formation of these products is associated with modification of DNA by NNK. The other major metabolic pathway was carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which detected both unconjugated and diastereomeric *O*-glucuronides. One of these glucuronides had been previously identified in rat urine, but the other diastereomer, which was the more prevalent of the two in serum and urine, had not been observed in studies of NNK metabolism in rodents. It was characterized by its spectral properties, by enzymatic hydrolysis to NNAL, and by derivatization of the released NNAL enantiomer with (*R*)-(+)-α-methylbenzylisocyanate. The two NNAL glucuronides accounted for 15–20% of the urinary metabolites in monkeys given 0.1 μg/kg NNK, which is similar to a smoker's dose, suggesting their use as dosimeters of NNK exposure in humans. Pharmacokinetic parameters were consistent with those observed in previous studies of nitrosamines, and varied predictably with body weight of five species. The results of this study have provided new insights relevant to assessing human metabolism of NNK.

Introduction

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) is a potent pulmonary carcinogen in rodents and is believed to play a role in the induction of lung cancer in smokers (1,2). NNK also causes tumors of the nasal mucosa, exocrine pancreas and liver in rats (1–3). The total doses of NNK to which smokers are exposed are similar to the lowest total doses that cause lung tumors in rodents (4,5). NNK and a related nitrosamine, *N'*-nitrosonornicotine, are the most abundant strong carcinogens present in unburned tobacco and are likely to be involved in the etiology of oral cancer in users of smokeless tobacco products (2).

Metabolic activation of NNK is a prerequisite for expression of its carcinogenicity. Extensive studies of NNK metabolism and DNA binding in rats and mice have conclusively demonstrated that α-hydroxylation (hydroxylation of the methylene and methyl carbon atoms adjacent to the *N*-nitroso group) is a key metabolic process leading to initiation of carcinogenesis (6–11). In addition, the overall pathways of NNK metabolism by α-hydroxylation, carbonyl reduction and pyridine-*N*-oxidation have been widely documented in rodent species, both *in vivo* and in various tissue preparations (1,6,7,12–14). However, only limited data are available on NNK metabolism in primates. In one study, Castonguay *et al.* (15) investigated the metabolism and distribution of NNK in marmosets. Whole body autoradiography as well as HPLC analyses were used to study the distribution of metabolites in two monkeys which were killed 4 h after injection of NNK. In a second study, Adams *et al.* (16) investigated the concentrations of NNK and its product of carbonyl reduction, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in the blood of a baboon at various time intervals after injection. There have been four reports of NNK metabolism in humans. In one, cultured human tissues were shown to metabolize NNK predominantly by carbonyl reduction, and to a limited extent by α-hydroxylation (17). A second study examined these pathways in human liver and lung microsomes (18). The presence of hemoglobin or DNA adducts, resulting from α-hydroxylation of NNK or *N'*-nitrosonornicotine in smokers or snuff-dippers, has been described (19,20).

The present study was undertaken to provide a more complete picture of the metabolism of NNK in primates. We were particularly interested in the extent to which the critical α-hydroxylation pathway occurred, and also wished to determine whether metabolites potentially suitable for human dosimetry studies could be identified. Therefore, we have investigated the metabolism of NNK in the patas monkey.

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; PBS, phosphate-buffered saline; NNAL-Gluc, [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid; NNAL-Carb [4-methylnitrosamino)-1-(3-pyridyl)but-1-yl]-*N*[(*R*)-α-methylbenzyl]carbamate; AUC, area under the curve; V_{ss}, volume of distribution at steady state; Cl, clearance; MRT, mean residence time; NNAL-*N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanol; NNK-*N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone; CI, chemical ionization.

Materials and methods

Chemicals

[5-^{3}H]NNK (2.12 Ci/mmol) was obtained from Chemsyn Science Laboratories, Lenexa, KS. It was diluted as necessary with unlabeled NNK, which was synthesized (21). Metabolite standards were synthesized (22) or isolated from urine of NNK-treated rats (23).

Apparatus

NMR spectra were determined on a Bruker AM 360 WB spectrometer. MS were obtained with a Hewlett-Packard model 5988A instrument. HPLC was carried out with a Waters Associates system (Millipore; Waters Division, Milford, MA) equipped with a model 440 UV/visible detector operated at 254 nm or a Flo-one/Beta radioactive flow detector (Radiomatic Instruments, Tampa, FL).

Patas monkey husbandry and treatment

Colony-reared female patas monkeys R154 (5 years old, 4.8 kg) and R113 (6 years old, 5.4 kg) were maintained in AAALAC-accredited housing in the Veterinary Resources Branch, Bethesda, MD. The characteristics and utilization of this species have been described previously (24). The monkeys were fitted with a collar and a Hydrocath (Access Technology Co., Skokie, IL) surgically implanted in the left femoral vein under anesthesia 2 or more weeks before experimentation. They were trained to accept chair restraint. Experiments 1–3 were carried out with R154 and experiments 4 and 5 with R113. For each experiment, the monkey was transferred to the chair and allowed to relax for at least 30 min before the beginning of treatment. Pieces of apple were given at intervals throughout the accommodation, treatment and collection periods. Both injections and blood samplings were by needle and syringe via the Hydrocath. Urine samples were collected in pans placed under the chair, or, after 6 h, under the cage. Urines were aliquoted for scintillation counting until negligible radioactivity was detected. Blood samples were centrifuged at 2000 r.p.m. for 30 min. Serum, urine and feces samples were frozen on dry ice for shipment.

In experiment 1, monkey R154 received ~900 μCi (18 μg/kg) [5-^{3}H]NNK in 5.4 ml sterile phosphate-buffered saline (PBS). In experiment 2, carried out after an interval of 2 months, the monkey, now weighing 6.1 kg, received 0.1 μg/kg [5-^{3}H]NNK in 2 ml PBS. One day later experiment 3 was carried out by injection of 0.2 mCi (4.9 mg/kg) [5-^{3}H]NNK. For experiment 4, monkey R113 was treated with 0.1 μg/kg [5-^{3}H]NNK in 2 ml PBS. After 24 h, experiment 5 was carried out by injection of ~900 μCi (16 μg/kg) [5-^{3}H]NNK.

Analysis of metabolites

Serum was purified by ultrafiltration using an Amicon Centrifree microparution system (Amicon Divison, W.R.Grace and Co., Beverly, MA), prior to HPLC analysis. All urine samples from experiments 1 and 2 were purified by ultrafiltration with the same system. The urine samples from experiments 4 and 5 required a preliminary purification step prior to ultrafiltration. This consisted of addition of a 10-fold excess of methanol to a measured volume of the sample. The sample was kept at −20°C for ~2 h, and then centrifuged to remove the precipitate. The supernatant was evaporated to dryness under N_2. One milliliter of H_2O was added to the residue and the sample was sonicated prior to ultrafiltration. Some urine samples, especially from the lowest dose experiments, required concentration prior to HPLC analysis. This was accomplished by freeze-drying a measured volume of the sample using a LabConco Lyph Lok 6 freeze dryer (LabConco, Kansas City, MO). After addition of 0.5–3 ml of H_2O, the sample was sonicated and then purified as already described.

The feces were dried by placing them in a freeze dryer for 12 h. They were then pulverized using a glass rod and freeze dried for 2 h. This was repeated twice before freeze drying them overnight. The samples were then extracted by sonication with 20–60 ml of methanol, three or four times. The methanol extracts were combined and themselves extracted with hexane three or four times. The methanol was evaporated to dryness and the residue was sonicated three times with 2 ml of 0.1 N HCl. Two milliliters of H_2O were added to the aqueous layer and the pH was adjusted to 7.0 with 1 N NaOH. The samples were further purified by ultrafiltration prior to HPLC analysis.

All samples were analyzed by reverse-phase HPLC using a Whatman 5 μm Partisphere C-18 cartridge column (4.6 × 250 mm, Whatman Co., Clifton, NJ). The initial solvent was 20 mM sodium phosphate buffer, pH 7.0. The elution program consisted of addition of methanol at a rate of 0.5% per min. When the solvent composition reached 8% methanol, the program was run isocratically for 15 min before continuing the addition of methanol. The metabolites were initially identified by their coelution with non-radioactive standards. The identities of the hydroxy acid and keto acid were confirmed by HPLC analysis using a Whatman 5 μm Partisphere C-18 column. The initial solvent in this system was 60 mM sodium acetate buffer, pH 4.5. Methanol was added according to the elution program used in the metabolite analysis above. Under these conditions, the retention times of hydroxy acid and keto acid were 18 and 43.5 min respectively.

Treatment of metabolites with β glucuronidase

NNAL was released from the glucuronides by treatment with β-glucuronidase, type IXA from *Escherichia coli* (Sigma Chemical Co., St. Louis, MO). [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-O-D-glucosiduronic acid [NNAL-Gluc(I)] was treated with 250–500 units at room temperature for 30–60 min to effect complete hydrolysis. NNAL-Gluc(II) required treatment with 250–500 units for 24 h at 37°C. To determine the extent of hydrolysis, the enzyme was removed by ultrafiltration and the samples were analyzed for NNAL by HPLC using a Phenomenex C-18 Bondclone column (source). The elution program was isocratic, with a 1 ml/min flow of 3/1:H_2O/methanol.

Preparation of [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-N(R)-α-methylbenzyl) carbamate [NNAL-Carb(I) and (II)]

To a solution of racemic NNAL (6.3 mg, 30 μmol) in 5 ml benzene was added triethylamine (170 μmol) and (*R*)-(+)-α-methylbenzylisocyanate (270 μmol, Aldrich Chemical Co., Milwaukee, WI). The reaction mixture was heated under reflux overnight, after which the benzene was evaporated. Methylene chloride was added and the mixture was purified on a silica gel column eluted with ethyl acetate. NNAL-Carb(I) and (II) were obtained as a mixture, ^{1}H NMR δ 8.60 (2H, bs, pyr-H_2, H_6), 7.55 (1H, m, pyr-H_4), 7.3 (6H, m, pyr-H_5 and PhH), 5.70 (1H, m, C*H*OH), 5.2 (0.7H, m, NH), 4.80 (1H, m, C*H*CH$_3$), 4.2 (1.6H, m, CH_2-N-N=O, E-isomers), 3.45–3.75 (0.9H, m, CH_2-N-N=O, Z-isomers and CH_3-N-N=O, Z-isomers), 3.01 [1.1H, s, CH_3-N-N=O, E-NNAL-Carb(I)], 2.96 [1.2H, s, CH_3-N-N=O, E-NNAL-Carb(II)], 1.6–2.0 (4H, m, -CH_2CH_2-), 1.50 [1.4H, d, CH_3CH, NNAL-Carb(I)], 1.45 [1.65H, d, CH_3CH, NNAL-Carb(II)]; MS, *m/z* (relative intensity), CI, 357 (M$^+$1, 5.5), 328 (2), 210 (17), 163 (9), 105 (100); EI, 326 (0.5), 191 (12), 161 (98), 132 (82), 105 (100), 84 (78).

Derivatization of [5-^{3}H]NNAL to [5-^{3}H]NNAL-Carb(I) and (II)

Metabolically formed [5-^{3}H]NNAL-Gluc(I) and (II) were treated with β-glucuronidase as described above, and the released [5-^{3}H]NNAL was collected from HPLC. Metabolically formed [5-^{3}H]NNAL was also collected. These collections were performed on the Phenomenex column as described above. Approximately 0.2 μmol of non-radioactive racemic NNAL was added to the collected material and the samples were evaporated to dryness. After addition of 5 ml benzene, 350–500 μmol of triethylamine and 140 μmol of (*R*)-(+)-α-methylbenzylisocyanate were added. The solutions were heated under reflux overnight, after which the benzene was evaporated, and $CHCl_3$ was added.

HPLC analysis of NNAL-Carb(I) and (II)

The diastereomeric carbamates of NNAL were separated by HPLC using an Alltech 5 μm Econosil silica column (Whatman, Inc., Clifton, NJ). The composition of the solvent system was 68.6% hexane, 29.4% $CHCl_3$ and 2.0% methanol. Elution was accomplished under isocratic conditions at a flow rate of 1 ml/min. The [5-^{3}H]NNAL carbamates were usually coinjected with the standard mixture of NNAL-Carb(I) and (II). The HPLC eluent from these analyses was collected at 0.5 min intervals using an Isco retriever III fraction collector (Isco Inc., Lincoln, NE). The solvent was evaporated and scintillation cocktail (Picofluor 40, Packard Instrument Co., Meriden, CT) was added. The radioactivity was measured using a Beckman LS 9800 liquid scintillation counter (Beckman Instruments, Irvine, CA).

Pharmacokinetic analyses

The serum concentration versus time data were analyzed using SIPHAR (Simed, Creteil, France), a pharmacokinetic parameter estimation program. The goodness-of-fit was determined by visual inspection of both the concentration versus time profile and the plot of the residuals. The area under the curve (AUC) from time zero to the last sampling time was determined using the trapezoidal rule, and the extrapolated area was calculated by dividing the concentration at the final time point by the apparent elimination rate constant. This extrapolated area was at most 30% of the total AUC. The half-times were estimated by unweighted linear regression of the log-transformed data. The volume of distribution at steady state (V_{ss}), clearance (Cl) and mean residence time (MRT) for NNK were determined using non-compartmental methods (25).

For interspecies extrapolation using the data obtained in these experiments and those found in the literature, interspecies scaling plots were made. By using the allometric equation $y = aB^x$ where B is body weight and x and a are the allometric exponent and coefficient, respectively, an equation can be obtained in which the body weights of different species can be related to physiological parameters shared by that group. By plotting a specific parameter as a function of body weight on a log–log plot a line can be fitted to the points with acceptable correlation. The parameters a and x were estimated by performing weighted ($1/y^2$) non-linear regression analyses using RS/E (BBN Software, Cambridge, MA).

Results

Clearance and excretion of NNK and its metabolites

In each of two female patas monkeys treated with ~16–18 μg/kg [5-^{3}H]NNK by i.v. injection (experiments 1 and 5), urine was the major route of excretion, although quantitation of the amount of radioactivity excreted was complicated by some losses during injection and by spraying of the urine. Approximately 50% of the radiolabel could be accounted for in the 24 h urine of the two monkeys. Less than 1% of the radiolabel could be extracted from feces with ethyl acetate.

Serum and urine samples were analyzed by HPLC. A typical chromatogram of serum metabolites is illustrated in Figure 1. This chromatogram was obtained from blood drawn 30 min after injection (experiment 1). The radioactive peaks were identified by coelution with standards. The major metabolites observed in this chromatogram were hydroxy acid, keto acid and NNAL (see

Figure 2). Minor metabolites were identified as diol 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanol (NNAL-*N*-oxide), and NNAL-Gluc(I). The latter was the same as NNAL-Gluc detected in the urine of rats and mice treated with NNK (23). The peak marked with an asterisk had not been previously detected in studies of rodent metabolism of NNK. It was identified by the data described below as NNAL-Gluc(II).

The serum concentration versus time profiles for NNK and selected metabolites are shown in Figure 3, and pharmacokinetic parameters are listed in Table I. NNK was cleared rapidly from the blood in both experiments, with MRTs of 14 min. The V_{ss} were 2081.0 and 1431.1 ml/kg for experiments 1 and 5 respectively. The shapes of the profiles of NNK in the two experiments were similar (Figure 3), although it achieved a 3-fold higher initial concentration in experiment 5, with a correspondingly lower Cl (55.1 versus 143 ml/min/kg).

To determine whether the clearance of NNK varies predictably with body weight, data from the present study and from the literature (16) were pooled and Cl values fitted to the allometric equation (Figure 4). The equation resulting from a non-linear regression fit is $Cl = 44.2B^{1.08}$ where B is body weight. Since the exponent was close to 1, a weighted ($1/y^2$) linear regression was performed. The equation obtained from this fit is $Cl = 53.1B - 0.806$. For a 70 kg human, Cl for NNK is estimated by extrapolation using the allometric equation to be 4346 ml/min.

The major metabolic products—NNAL, hydroxy acid and keto acid—as well as diol, achieved significant serum concentrations that were maximal shortly after injection. C_{max} values for NNAL and diol were similar in the two experiments, whereas hydroxy and keto acids achieved a somewhat greater maximum in experiment 1. All disappeared rather rapidly and were mostly gone within 2 h; this process was somewhat slower in experiment 1 compared with experiment 5, as indicated by the higher AUC and terminal $t_{1/2}$ values. NNAL-*N*-oxide presented essentially identical profiles for the two experiments; 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone (NNK-*N*-oxide) was detected only in experiment 5. NNAL-Gluc(I) achieved lower C_{max} and AUC and greater $t_{1/2}$ values than NNAL-Gluc(II) in both experiments, with C_{max} for NNAL-Gluc(I) being reached at 45 min and NNAL-Gluc(II) at 10 min. There was more rapid disappearance of both in experiment 5 compared with experiment 1.

A typical chromatogram of urinary metabolites, from a sample collected 145–390 min after injection (experiment 1), is illustrated in Figure 5. The major metabolites were hydroxy acid, keto acid and the peak marked with the asterisk, which is the same as the corresponding peak in Figure 1. Minor metabolites were NNAL-*N*-oxide, NNAL-Gluc(I) and NNK-*N*-oxide. In addition, several minor peaks not corresponding in retention time to any known metabolites were consistently observed.

The amounts and percentages of the metabolites detected in urine are shown in Table II. The metabolites in experiment 1 were contained in a total of 857 ml urine, of which 49 ml were excreted within 7 h after treatment; for experiment 5 there were 1184 ml urine, with 19.5 ml in the first 7 h. The total of metabolites and the relative proportions of each were quite similar in the two experiments. However, the relative amounts of some metabolites in urine changed somewhat over time (data not

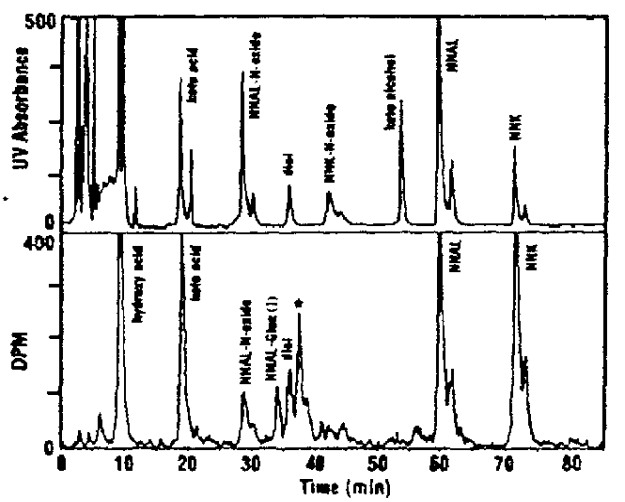


Fig. 1. Chromatogram obtained upon HPLC analysis of serum drawn from a patas monkey 30 min after i.v. injection of [5-^{3}H]NNK (experiment 1). The peak with the asterisk was identified as NNAL-Gluc(II) by the data described in the text.

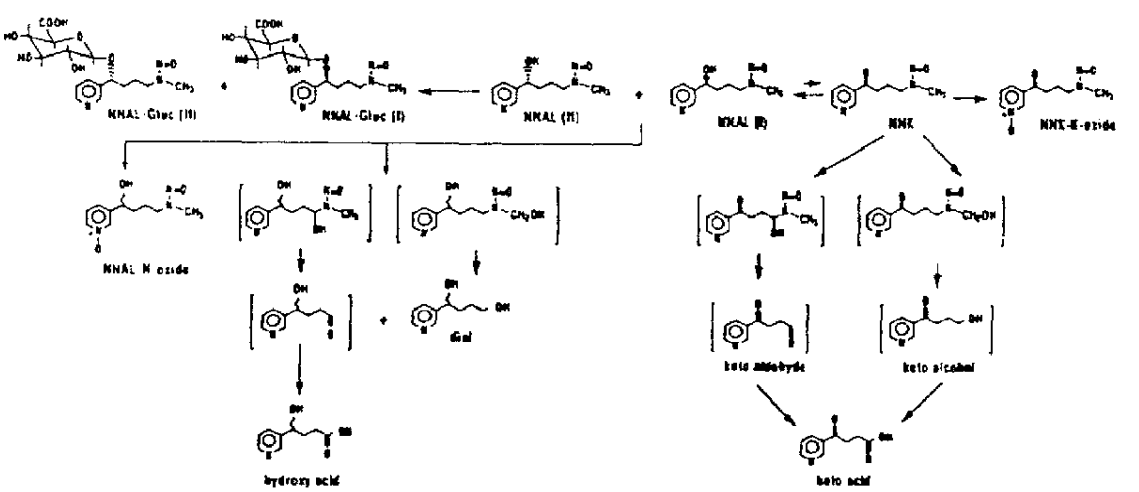


Fig. 2. Metabolism of NNK in the patas monkey. Structures in brackets were not detected in serum or urine but are implied intermediates based on previous metabolism studies in rodents.

shown). In both experiments, the relative amounts of keto acid were greater in the first 1–2 h after dosing, and NNAL and NNAL-*N*-oxide less, than in the 24 h urine.

Identification and characterization of NNAL-Gluc(II)

Since the peak with the asterisk in Figures 1 and 5 appeared to be a previously undetected metabolite of NNK, we carried out further experiments aimed at its characterization. The peak was collected from the urine and serum samples and incubated with β-glucuronidase. Analysis of the resulting mixture by HPLC revealed that ~90% of the unknown peak had been converted to new peaks, which coeluted with the E- and Z-isomers of NNAL. The conversion of this peak to NNAL by incubation with β-glucuronidase occurred more slowly than the corresponding hydrolysis of NNAL-Gluc(I). These data suggested that the new metabolite might be a diastereomer of NNAL glucuronide. This is possible because NNK has a prochiral center at its carbonyl group, and reduction can give either of the NNAL enantiomers—I and II—illustrated in Figure 2. Conjugation with β-D-glucuronic acid would produce the diastereomers—NNAL-Gluc(I) and NNAL-Gluc(II). In order to obtain spectral data on the unknown metabolite, a patas monkey was treated with 4.9 mg/kg [5-^{3}H]NNK (experiment 3), and the urinary metabolites were separated by HPLC. The distribution of urinary metabolites was similar to that presented in Table II. The unknown peak was purified by collection from HPLC and its ^{1}H and ^{13}C NMR spectra were determined. The spectral data and those for NNAL-Gluc(I) isolated from the urine of NNK-treated rats are summarized in Tables III and IV (23). Assignments were confirmed by decoupling experiments. The proton spectra were remarkably similar, except for the chemical shift of proton 1′ which appeared ~0.5 p.p.m. further upfield in the spectrum of the new metabolite than in the spectrum of NNAL-Gluc(I). This proton overlapped with protons *d* in the spectra run in D_2O, but when the spectra were run in [d_4]MeOH, they were resolved. The carbon spectra were also similar, with the main differences being found in the chemical shifts of carbons *a* and 1′.

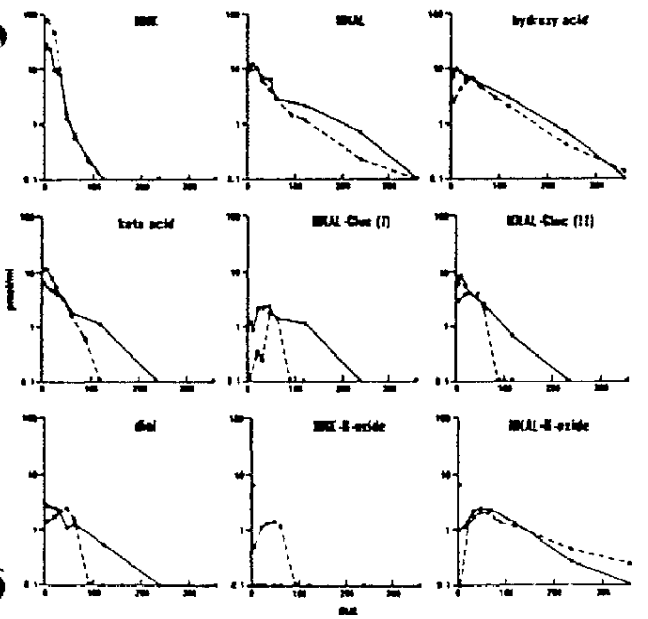


Fig. 3. Time course of [5-^{3}H]NNK and metabolites in patas monkey serum after i.v. injection of [5-^{3}H]NNK (●——●) experiment 1, (○- - -○) experiment 5

The MS of this sample, obtained by desorption chemical ionization (CI), showed an M+1 peak at *m/z* 390, which is expected for NNAL-Gluc(II), molecular weight 386, in which the four exchangeable protons of the sugar ring are deuterium due to exchange with D_2O. These NMR and MS data were entirely consistent with the assignment of the new metabolite as NNAL-Gluc(II).

In order to obtain more information on the stereoselectivity of NNAL glucuronide formation, and to confirm our structural assignment, we resolved the enantiomers of NNAL released by hydrolysis of NNAL-Gluc(I) and (II). Racemic NNAL was allowed to react with (*R*)-(+)-α-methylbenzylisocyanate to form a mixture of diastereomeric carbamates which were characterized by ^{1}H NMR. This mixture would contain four isomers consisting of two diastereomers of each E- and Z-isomer. Normal phase HPLC was employed to partially separate this mixture into three peaks as illustrated in the UV traces of Figure 6A. The second peak in these traces was a mixture of the E (major) isomer of NNAL-Carb(II) and the Z (minor) isomer of NNAL-Carb(I). Then NNAL-Gluc(I) and NNAL-Gluc(II) from patas monkey urine were each treated with β-glucuronidase, and the released NNAL purified by HPLC and reacted with (*R*)-(+)-α-methylbenzylisocyanate. Analysis of the products gave the chromatograms illustrated in Figure 6A and B. NNAL-Gluc(I) released NNAL which was converted to NNAL-Carb(I) whereas NNAL-Gluc(II) released NNAL which was converted to NNAL-Carb(II). Then, NNAL-Gluc(I) from rat urine (23) was subjected to the same procedure. The results of this analysis are illustrated in Figure 6C, and are consistent with the presence of only one

Table I. Pharmacokinetic parameters for NNK and metabolites[a]

	Experiment 1			Experiment 5		
	C_{max} (pmol/ml)	AUC (pmol·ml·min)	$t_{½}$ (min)	C_{max} (pmol/ml)	AUC (pmol·ml·min)	$t_{½}$ (min)
NNK	27.6	632	5.5	77.1	1490	18.0
Hydroxy acid	9.9	975	61.6	6.7	683	55.5
Keto acid	12.0	500	32.6	6.5	292	24.4
Diol	2.7	201	48.0	2.4	158	22.1
NNAL-*N*-oxide	2.4	342	58.0	2.1	360	100.0
NNK-*N*-oxide	–	–	–	1.4	182	67.4
NNAL-Gluc(I)	2.3	327	89.0	1.8	128	41.3
NNAL-Gluc(II)	8.6	412	31.3	4.4	265	18.2
NNAL	12.0	839	58.4	10.0	594	45.8

[a]Two female patas monkeys (4.8 and 5.4 kg) were given i.v. injections of ~18 or 16 μg/kg [5-^{3}H]NNK in experiments 1 and 5 respectively

diastereomer—NNAL-Gluc(I)—in rat urine. A coinjection of NNAL-Carb(I) from rat urine and NNAL-Carb(II) from patas monkey urine is illustrated in Figure 6D. These results confirm the structural assignments of NNAL-Gluc(I) and NNAL-Gluc(II). The absolute configurations of the NNAL enantiomers and diastereomers are not yet known; the configurations shown in Figure 2 are arbitrary.

The enantiomeric compositions of the unconjugated urinary NNAL peaks were also investigated. NNAL from patas monkey serum and urine formed a mixture of NNAL-Carb(I) and NNAL-Carb(II) (Figure 7A and B) while NNAL from rat urine appeared to form exclusively NNAL-Carb(I). These data are presented in Figure 7A–C.

Urinary metabolites from monkeys after a smoker's dose of NNK

Table V summarizes the results of analyses of urine from two monkeys injected with 0.1 μg/kg [5-^{3}H]NNK, which is the approximate dose of NNK to which a smoker of two packs of cigarettes per day is exposed, based on 135 ng NNK/cigarette

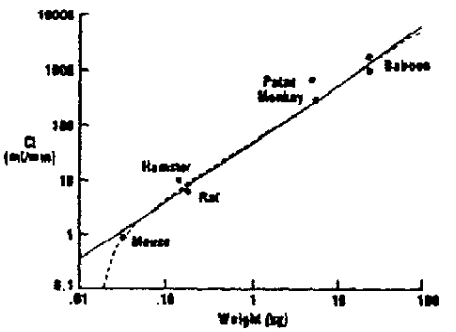


Fig. 4. Interspecies scaling of systemic NNK clearance as a function of body weight as determined from a weighted ($1/y^2$) non-linear regression using the allometric equation (dashed line) and from a weighted ($1/y^2$) linear regression (solid line). The 95% confidence intervals for the allometric equation $Cl = aB^x$ are $24 < a < 63.6$ and $-0.920 < x < 1.23$. The 95% confidence intervals for the parameters of the linear equation ($Cl = aB + C$) are $28.8 < a < 77.4$ and $-2.04 < C < 0.430$.

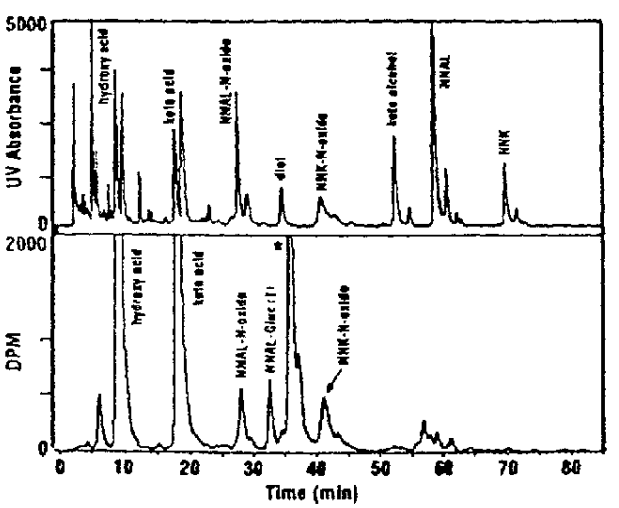


Fig. 5. Chromatogram obtained upon HPLC analysis of urine collected from 145 to 390 min after i.v. injection of [5-^{3}H]NNK (experiment 1). The peak with the asterisk was identified as NNAL-Gluc(II) by the data described in the text

in a popular US brand (K.D.Brunnemann and D.Hoffmann, unpublished data). The pattern of metabolites was similar in the two monkeys, and was also similar to that seen at the higher doses.

Discussion

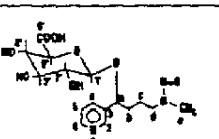
One goal of this study was to determine the qualitative pattern of NNK metabolism in a primate model. Previous studies in rodent models have shown that the products of NNK α-hydroxylation are major urinary metabolites, generally comprising at least 50% of the total (6,23,26,27). Other prominent urinary metabolites in rodents include NNAL, NNAL-Gluc(I), NNAL-*N*-oxide and NNK-*N*-oxide, although the levels of these are highly dependent on dose (23,26). Studies with cultured human tissues as well as human liver and lung microsomes have indicated that carbonyl reduction to NNAL

Table II. Urinary metabolites of NNK in the patas monkey (~16–18 μg/kg dose)[a]

Metabolite	nmol detected in urine (% of urinary metabolites)	
	Experiment 1	Experiment 5
Hydroxy acid	53.3 (44.0)	38.7 (41.7)
Keto acid	27.1 (22.4)	19.5 (21.0)
NNAL-*N*-oxide	10.3 (8.51)	8.79 (9.47)
NNAL-Gluc(I)	5.69 (4.70)	3.61 (3.89)
Diol	1.58 (1.31)	ND[b]
NNAL-Gluc(II)	17.9 (14.8)	18.0 (19.4)
NNK-*N*-oxide	4.96 (4.10)	4.03 (4.34)
NNAL	ND	ND
NNK	ND	0.165 (0.018)
Total	121 (100)	92.8 (100)

[a]Two female patas monkeys (4.8 and 5.4 kg) were given i.v. injections of ~18 or 16 μg/kg [5-^{3}H]NNK in experiments 1 and 5 respectively. Urine was collected for 24 h and analyzed by HPLC as described in Materials and methods.
[b]Not detected.

Table III. ^{1}H NMR assignments for NNAL-Gluc(I) and NNAL-Gluc(II)



Protons	NNAL-Gluc(I)[a]	NNAL-Gluc(II)
2	8.51 (s)	8.49 (s)
4	7.87 (dt)	7.86 (dt)
5	7.43 (dd)	7.45 (dd)
6	8.44 (d)	8.46 (d)
a	4.86 (Z),*t*; 4.91 (E),*t*	4.98 (m)
b,c	1.66-2.04 (m)	1.64-2.0 (m)
d	4.16 (t)	4.16 (m)[b]
e	3.04 (E),*s*; 3.69 (Z),*s*	3.05 (E),*s*; 3.68 (Z),*s*
1′	4.57 (E),*d*; 4.56 (Z),*d*	4.16 (m)[c]
2′	3.31 (t)	3.25-3.37 (m)
3′	3.43 (t)	
4′	3.43 (t)	3.48 (t)
5′	3.53 (d)	3.54 (d)

[a]Data from reference 23.
[b]Appears as a multiplet, 4.19–4.28 p.p.m. in spectra run in [d_4]MeOH.
[c]Appears as a doublet at 4.10 p.p.m. in spectra run in [d_4]MeOH

Table IV. ^{13}C NMR assignments for NNAL-Gluc(I) and NNAL-Gluc(II)

Carbons[a]	NNAL-Gluc(I)[b]	NNAL-Gluc(II)
2	151.1	151.3
3	140.0	NO[c]
4	139.0	139.1
5	126.9	127.1
6	150.3	150.5
a	82.2	79.7
b	35.2	36.9
c	25.4	25.5
d	57.0	56.9
e	34.5	35.1
1'	104.6	102.2
2'	76.1	75.6
3'	78.5	78.8
4'	74.7	74.6
5'	79.4	79.7
6'	178.3	178.4

[a]See structure in Table III.
[b]Data from reference 23.
[c]Not observed.

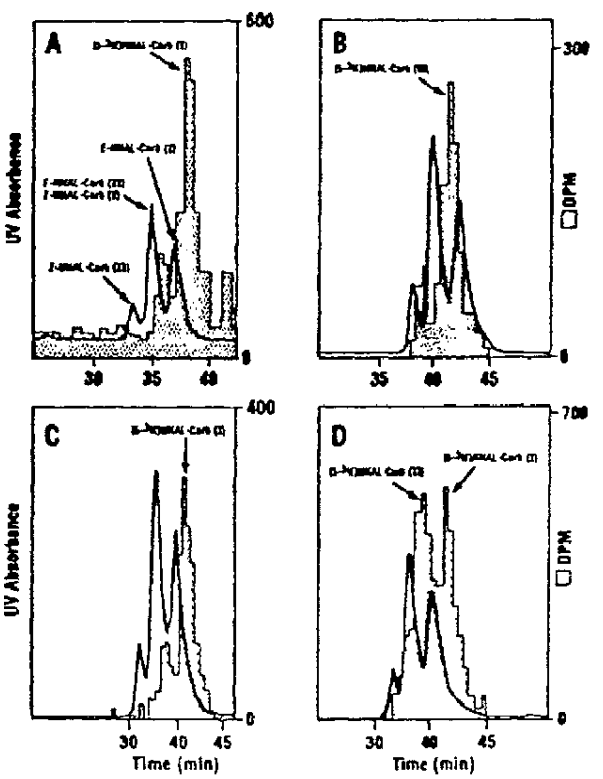


Fig. 6. HPLC analysis of NNAL-Gluc diastereomers in urine. The UV traces represent the E- and Z-isomers of NNAL-Carb(I) and NNAL-Carb(II), formed by reaction of NNAL(I) and NNAL(II) with (*R*)-(+)-α-methylbenzylisocyanate (see panel A). The histograms represent the d.p.m. associated with [5-^{3}H]NNAL-Carb(I) and [5-^{3}H]NNAL-Carb(II) formed by reaction with (*R*)-(+)-α-methylbenzylisocyanate of [5-^{3}H]NNAL(I) and [5-^{3}H]NNAL(II), released from their urinary glucuronides, as follows: (A) [5-^{3}H]NNAL-Gluc(I) from monkey urine, (B) [5-^{3}H]NNAL-Gluc(II) from monkey urine; (C) [5-^{3}H]NNAL-Gluc(I) from rat urine; and (D) conjection of [5-^{3}H]NNAL-Gluc(I) from rat urine and [5-^{3}H]NNAL-Gluc(II) from monkey urine.

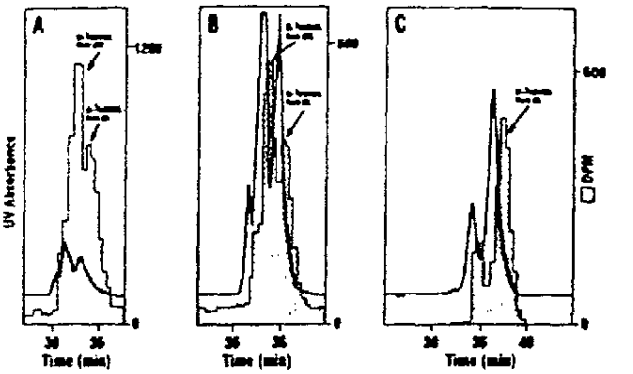


Fig. 7. HPLC analysis of [5-^{3}H]NNAL-Carb(I) and [5-^{3}H]NNAL-Carb(II) formed from unconjugated [5-^{3}H]NNAL in (A) monkey serum obtained 5 min after injection of [5-^{3}H]NNK (experiment 5); (B) monkey urine collected 245 min after i.v. injection of [5-^{3}H]NNK (experiment 5); and (C) rat urine collected after administration of [5-^{3}H]NNK (ref. 23).

Table V. Urinary metabolites of NNK in the patas monkey (0.1 μg/kg dose)[a]

Metabolite	nmol detected in urine (% of urinary metabol.	
	Experiment 2	Experiment 4
Hydroxy acid	137 (42.9)	65.3 (41.9)
Keto acid	81.5 (25.6)	38.4 (24.6)
NNAL-*N*-oxide	18.3 (15.7)	12.0 (7.69)
NNAL-Gluc(I)	11.5 (3.61)	5.6 (3.6)
Diol	n.d.[b]	n.d.[b]
NNAL-Gluc(II)	49.4 (15.5)	25.3 (16.2)
NNK-*N*-oxide	11.6 (13.6)	8.9 (15.7)
NNAL	6.48 (2.03)	ND[c]
NNK	3.02 (0.095)	ND[c]
Total	319 (100)	156 (100)

[a]Two female patas monkeys (4.8 and 5.4 kg) were given i.v. injections of 0.1 μg/kg [5-^{3}H]NNK in experiments 2 and 4 respectively. Urine was collected for 3 h and analyzed by HPLC as described in Materials and methods.
[b]Not determined due to overlapping peaks.
[c]Not detected.

greatly exceeds metabolism by α-hydroxylation (17,18). Nevertheless, DNA and hemoglobin adducts resulting from α-hydroxylation of NNK or *N*'-nitrosonornicotine have b detected in tissues or blood of smokers (19,20). Investigation of NNK metabolism in a primate model might provide some indication of the expected relative amounts of α-hydroxylation and carbonyl reduction *in vivo* in humans, although it should be noted that NNAL and NNK can be interconverted *in vivo* and that both can undergo α-hydroxylation (16,28). The results clearly demonstrate that α-hydroxylation, leading to hydroxy and keto acids, is a major metabolic pathway, as in the rodent. These products comprised at least 60% of the urinary metabolites. Approximately 30% of the urinary metabolites resulted from carbonyl reduction followed by glucuronidation or *N*-oxidation.

Cl values for a dose of ~90 nmol/kg NNK in the two patas monkeys, 55 and 143 ml/min/kg, were similar to those obtained with two baboons given 15 μmol/kg, 42 and 75 ml/min/kg (16). Values for the $t_{½}$ in these baboons, 25.2 and 19.2 min, were similar to the terminal phase $t_{½}$ in experiment 5 of our study, 18 min. Thus, for at least two species of primate, metabolic conversion of NNK is rapid. The prominence among the metabolites of the keto and hydroxy acids indicates that the brisk

metabolism of NNK in these primates entails efficient production of ultimate carcinogenic forms.

Cl (73 ml/min/kg) and $t_{1/2}$ (14.4 min) in Syrian golden hamsters given 150 μmol/kg NNK were similar to those observed here (16). By contrast, rats and mice had lower Cl (29 – 38 and 28 ml/min/kg respectively) (16). A log – log plot of Cl versus body weight for five species treated with NNK resulted in a straight line (Figure 4). Thus, Cl for NNK varied predictably with body weight, as was the case for *N*-nitrosodimethylamine (29). Low molecular weight compounds have been noted to deviate from the more general finding of reduced Cl with increasing body weight (30).

The allometric equations for NNK, $Cl = 44.2B^{1.08}$, and for *N*-nitrosodimethylamine, $Cl = 49.7B^{0.998}$ (29), were remarkably similar; measured Cl values in patas monkeys and extrapolated Cl for humans for the two compounds were in the same range. This may imply that common phenomena govern the pharmacokinetics of these nitrosamines.

The stereoselectivity of NNAL formation is apparently different in the rat and patas monkey. Analysis of NNAL from rat urine demonstrated the presence of one enantiomer, NNAL(I), whereas both enantiomers were detected in patas monkey urine and serum. Consistent with this finding, only one diastereometric glucuronide—NNAL-Gluc(I)—was detected in rat urine whereas both NNAL-Gluc(I) and NNAL-Gluc(II) were found in patas monkey urine and serum. This may be due to differing extents of stereoselectivity of carbonyl reduction in the rat and patas monkey, or it could result from preferential metabolism in the rat of NNAL(II) by α-hydroxylation or *N*-oxidation. The rates of glucuronidation of NNAL(I) and NNAL(II) may also differ in the rat and monkey. Enantiomer selectivity in glucuronidation has been observed previously, and is known to be species dependent (31,32). These aspects require further investigation, as does assignment of the absolute configurations of NNAL(I) and NNAL(II).

NNAL-Gluc(I) and NNAL-Gluc(II) comprised 15 – 25% of the urinary metabolites of NNK at all doses examined. In the rat, urinary concentrations of NNAL-Gluc(I) decreased with dose, leading Morse *et al.* (23) to speculate that it would not be a good dosimeter for human uptake of NNK because it was not detected at the lowest dose examined, which was still at least 10 times as great as the expected dose in a smoker. However, the results of this study suggest that the NNAL-Gluc diastereomers may in fact be suitable markers for NNK exposure because they were readily detectable at all doses of NNK including 0.1 μg/kg. We are presently analyzing smokers' urine for NNAL-Gluc(I) and NNAL-Gluc(II).

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